

PATENT

Our Docket: P-ZA 3519

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

|                          |   |                        |
|--------------------------|---|------------------------|
| In re application of:    | ) |                        |
| Maurizio Zanetti         | ) | Group Art Unit: 1632   |
|                          | ) |                        |
| Serial No.: 09/300,959   | ) | Examiner: A. Beckerleg |
|                          | ) |                        |
| Filed: April 27, 1999    | ) |                        |
|                          | ) |                        |
| For: SOMATIC TRANSGENE   | ) |                        |
| IMMUNIZATION AND RELATED | ) |                        |
| METHODS                  | ) |                        |
|                          | ) |                        |

Commissioner for Patents  
Washington, D.C. 20231

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, the undersigned, declare as follows:

1) I am the Maurizio Zanetti who is named as the inventor on the above-identified patent application.

2) I understand that the claims stand rejected, in part, as allegedly lacking enablement for treating a variety of diseases and conditions.

Administration of nucleic acids Intrasplenically induces protective immunity against influenza A virus infection

3) The class I-restricted peptide NP<sub>366-374</sub> (ASNENMETM) of influenza A virus is immunodominant and induces protective immunity in mice. Plasmid  $\gamma$ 1NP<sup>3</sup> was created by inserting the NP<sub>366-374</sub> polypeptide into the third complementarity-determining region (CDR) of the murine IgH heavy chain gene V<sub>H</sub>62 and cloning

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the resulting V region into the expression vector pSVneoY1 coding for the human IgG1 constant region under the control of the IgH promoter and enhancer elements. Plasmid  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> is identical to  $\gamma$ 1NP<sup>3</sup> except that it also contains the heterologous Th cell determinant, NANPNVDPNANP inserted in CDR2. Mice were immunized with 100  $\mu$ g of plasmid in PBS by single intra-spleen inoculation.

4) As shown in Exhibit 1, mice immunized *in vivo* with plasmid  $\gamma$ 1NP<sup>3</sup> or plasmid  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> developed cytotoxic T lymphocytes (CTL) specific for NP<sub>366-374</sub>. Twenty one days after immunization with plasmid  $\gamma$ 1NP<sup>3</sup> (Group II), 66% of mice developed CTL specific for NP<sub>366-374</sub>, comparably better than the levels induced at the same time for mice immunized with synthetic NP<sub>366-374</sub> peptide (Group I). Further improvement in CTL induction was observed 21 days after immunization with plasmid  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> (Group III), with 93% of mice developing CTL specific for NP<sub>366-374</sub>. Moreover, as shown in the last column of Exhibit 1, responders immunized with the  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> had, on average, a two fold higher percent specific lysis than responders immunized with synthetic NP<sub>366-374</sub> peptide.

5) As shown in Exhibit 2, *in vivo* immunization with  $\gamma$ 1NP<sup>3</sup> provided protection against acute infection from influenza virus. Mice (10 per group) were immunized either with a single intraspleen inoculation of  $\gamma$ 1NP<sup>3</sup> (closed diamonds), intraspleen inoculation of  $\gamma$ 1NP<sup>3</sup> followed by subcutaneous immunization with

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synthetic peptide NP<sub>366-374</sub> (closed triangles), or two subcutaneous immunizations of synthetic peptide NP<sub>366-374</sub> (X). Untreated mice (open squares) served as a control. All mice were infected by intranasal inoculation with a 10xLD<sub>50</sub> infectious dose of A/PR8/34 (MA) influenza virus 180 days after priming. All non-vaccinated mice died by day 7, all mice immunized with control plasmid pSVneoY1 died by day 11, and all peptide-immunized mice died by day 11. In contrast, 60% of mice immunized with plasmid  $\gamma$ 1NP<sup>3</sup> followed by NP<sub>366-374</sub> booster and 50% of mice immunized with plasmid  $\gamma$ 1NP<sup>3</sup> alone resisted virus challenge for the 30 day observation period.

6) As shown in Exhibit 3, *in vivo* immunization with  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> protected mice against acute infection when challenged with very high doses of influenza virus. Mice (7-10 per group) were immunized either by intraspleen inoculation of  $\gamma$ 1NP<sup>3</sup> (closed diamonds),  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> (closed squares),  $\gamma$ 1NP<sup>3</sup> $\Delta$ L11 (closed triangles), or with two subcutaneous immunizations of synthetic peptide NP<sub>366-374</sub> (X). Untreated mice (open squares) served as a control. All mice were infected with intranasal inoculation of a 20xLD<sub>50</sub> infectious dose of A/PR8/34 (MA) influenza virus 80 days after priming. All non-vaccinated and all peptide-immunized mice died within 9 days of challenge. Mice vaccinated with the leader sequence deletion  $\gamma$ 1NP<sup>3</sup> $\Delta$ L11 plasmid also died by day 9. In contrast, vaccination using the dual epitope  $\gamma$ 1NV<sup>2</sup>NP<sup>3</sup> plasmid afforded protection in 60% of mice after 28 days and  $\gamma$ 1NP<sup>3</sup> plasmid-vaccinated mice were marginally protected.

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Administration of ex vivo transfected cells induces protective immunity against influenza A virus infection

7) CTL responses were observed in mice immunized with transgenic  $\gamma 1NV^2NP^3$  spleen lymphocytes. C57B1/6 spleen lymphocytes were transfected ex vivo with plasmid  $\gamma 1NV^2NP^3$ , and  $1 \times 10^3$  to  $2 \times 10^4$  of the transgenic spleen cells were intravenously administered to C57B1/6 mice. In three independent experiments, a specific and in most instances strong CTL response was measured in 39 out of 39 (100%) mice starting from day 14.

8) As shown in Exhibit 4, ex vivo immunization with transgenic  $\gamma 1NV^2NP^3$  spleen lymphocytes provided complete protection against acute lethal infection from influenza virus. C57B1/6 mice were intravenously immunized with transgenic spleen lymphocytes and challenged after twenty one days with a lethal intranasal dose of influenza virus. All control mice inoculated with syngeneic lymphocytes transgenic for plasmid pSVneoY1 (open squares) died by day 9. In contrast, all mice vaccinated with lymphocytes transgenic for  $\gamma 1NV^2NP^3$  resisted the challenge and survived for the full 40 day observation period.

9) As shown in Exhibit 5, administration of as few as 300 lymphocytes transgenic for  $\gamma 1NV^2NP^3$  protected mice from lethal influenza A virus infection. In contrast, mice vaccinated with lymphocytes transgenic for control plasmid  $\gamma 1NV^2NA^3$ , identical to  $\gamma 1NV^2NP^3$  with the exception of containing the antigen of

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*Plasmodium falciparum* in place of the Influenza Virus NP<sub>366-374</sub> peptide, were not protected from lethal influenza A infection.

Administration of nucleic acids Intrasplenically induces protective immunity against experimental allergic encephalomyelitis

10) Experimental allergic encephalomyelitis is a rodent model of multiple sclerosis. The T cell receptor (TCR) of autoreactive T lymphocytes, specific for myelin basic protein, mediates the pathogenesis of the disease. Plasmids expressing a TCR vaccine were prepared as follows. The  $\gamma$ 1TCRI plasmid was produced by inserting the rat Vb8.2 CDR3 peptide (ASSDSSNTE) into CDR3 of an IgH heavy chain gene and the  $\gamma$ 1TCRII plasmid was produced by inserting the rat Vb8.2 CDR2 peptide (DMGHGLRLIHYSYDVNSTEKG) into CDR3 of an IgH heavy chain gene. A schematic representation of the modified H chains is shown in Exhibit 6. Plasmid construction and intra-spleen inoculation were performed using methods similar to those described in paragraph 3.

11) As shown in Exhibit 7, the incidence of experimental allergic encephalomyelitis was prevented in rats inoculated with  $\gamma$ 1TCRII. Furthermore, the severity of disease (disease index) was markedly reduced for rats inoculated with  $\gamma$ 1TCRII plasmid. Similarly, rats given both the  $\gamma$ 1TCRI and  $\gamma$ 1TCRII plasmids were also protected.

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12) Exhibit 8 shows the distribution of experimental allergic encephalomyelitis according to severity score. Only 45% of rats receiving  $\gamma$ 1TCRII (Groups II and III) developed severe experimental allergic encephalomyelitis (score  $\geq 3$ ), significantly fewer than the 87% of non-inoculated control rats (Group VI) or 83% of rats inoculated with the  $\gamma$ 1WT control plasmid (Group V). Furthermore, none of the rats receiving  $\gamma$ 1TCRII had a severity greater than 3.

13) Exhibit 9 shows that the average duration of severe disease, in  $\gamma$ 1TCRII-inoculated rats, was shorter than in any other group. Notably, only one rat developed experimental allergic encephalomyelitis with a severity score of 3 lasting more than 2 days. In control rats, 13 of 16 (81%) had a duration of experimental allergic encephalomyelitis in grade 3 for 3 days or longer (data not shown).

Administration of ex vivo transfected cells induces protective immunity against B16-MUC.1 tumors

14) Mucin 1 is a glycoprotein expressed in tumors of epithelial origin in humans. C57B1/6 mice are non-tolerant of mucin 1, while MUC.1 transgenic mice, expressing the glycoprotein, are mucin 1 self tolerant. Immunization of mice with mucin 1 was carried out with plasmid  $\gamma$ 1NV<sup>2</sup>VTSA<sup>3</sup>, which expresses an IgH heavy chain containing a MUC-1 epitope in CDR3 and a Th cell determinant, NANPNVDPNANP in CDR2 under the control of IgH promoter and enhancer elements. Plasmid  $\gamma$ 1NV<sup>2</sup>NA<sup>3</sup> is

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identical to  $\gamma 1NV^2VTSA^3$  with the exception of containing the antigen of *Plasmodium falciparum* in place of the MUC-1 epitope. C57B1/6 spleen lymphocytes were transfected *ex vivo* and administered to mice using methods similar to those described in paragraph 7.

15) As shown in Exhibit 10, CD4 T cell proliferation was induced in both C57B1/6 (non-tolerant) mice and MUC.1 transgenic (tolerant) mice immunized with transgenic  $\gamma 1NV^2VTSA^3$  lymphocytes. The response was against both the dominant and sub-immunogenic MUC-1 determinant and was comparable to the response induced in non-tolerant C57B1/6 mice. For spleen lymphocytes harvested two weeks after immunization, the responses were significantly higher than observed for control immunizations with  $\gamma 1TL540$  lymphocytes expressing a determinant of human telomerase reverse transcriptase.

16) As shown in Exhibit 11, mice immunized with transgenic  $\gamma 1NV^2VTSA^3$  lymphocytes were protected from tumor formation. MUC.1 transgenic mice were immunized with lymphocytes transgenic for either  $\gamma 1NV^2VTSA^3$  (closed squares),  $\gamma 1NV^2NA^3$  (closed triangles) or pSVneo (open squares), receiving a first immunization of  $5 \times 10^3$  transgenic lymphocytes on day 0 followed by a similar immunization on day 21. Mice were challenged three days after the second immunization with  $2 \times 10^4$  B16-MUC.1 melanoma tumor cells or B16 neo melanoma tumor cells subcutaneously. Control mice immunized with non transgenic lymphocytes (open triangles) were also challenged. The number of mice in each group is indicated in parenthesis in Exhibit 11. All 16 mice

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vaccinated with transgenic  $\gamma 1NV^2VTSA^3$  lymphocytes and challenged with B16-MUC.1 tumor cells were protected, surviving through the 8 month observation period tumor-free. In contrast, all mice vaccinated with control transgenic lymphocytes developed tumors and expired before 30 days. Mice vaccinated with lymphocytes transgenic for  $\gamma 1NV^2VTSA^3$  and challenged with the B16 neo tumor cells lacking the mucin 1 antigen (asterisks) also formed tumors and died, indicating that the protection against B16-MUC.1 tumor challenge was specific for the mucin 1 antigen.

17) As shown in Exhibit 12, a subsequent challenge of B16-MUC.1 protected mice with tumor cells demonstrated that protection was long lived and specific. To this end, 70 days after the first challenge with B16-MUC.1 tumor cells, 12 protected mice (immunized with transgenic  $\gamma 1NV^2VTSA^3$  lymphocytes) were divided into two groups and challenged again with either B16-MUC.1 (closed squares) or B16 neo (open squares) tumor cells. Mice re-challenged with the MUC.1-expressing tumor continued to be protected. In contrast, mice that received B16 neo tumor cells died like controls that had been immunized with nontransgenic lymphocytes (open triangles).

18) In summary, treatment of a variety of pathological conditions using the methods of the invention has been demonstrated. In addition, to those exemplified in the specification, the results described above show stimulation of an immune response for treatment of influenza A virus infection, encephalomyelitis and cancer. The results described above also



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demonstrate successful *in vivo* and *ex vivo* administration of nucleic acids.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Date: 11/08/02

By:   
Maurizio Zanetti